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Targeting of Cationic Liposomes to Skin-Associated Bacteria

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Abstract: Cationic vesicles were produced by incorporating positively-charged stearylamine into the lipid bilayers. The addition of stearylamine has been shown to facilitate targeting of liposomes to the skin-associated bacterium *Staphylococcus epidermidis*. The adsorption of the liposomes to films of the bacteria immobilized on a solid surface has been studied. The extent of adsorption as a function of liposomal lipid concentration could be described in terms of a Langmuir adsorption isotherm, applicable to situations in which species are adsorbed as monolayers on solid surfaces. Analysis on the basis of the Langmuir model enabled the determination of the maximum theoretical targeting to the bacteria and association/dissociation constants for the interaction. The adsorption was examined under conditions of varying ionic strength. Increasing the ionic strength had the effect of decreasing the extent of targeting and suggested that the interaction between the cationic vesicles and sites on the bacterial surface was mediated by electrical double-layer effects. It follows that electrostatic effects make a major contribution to the interaction between these vesicles and *S. epidermidis*. The attachment of the vesicles is reversible and ionic strength-induced removal of vesicles from the bacteria could be used as a tool to study the delivery of liposomally encapsulated bactericide to the biofilm.

Key words: Visicles, adsorption, bacteria, *staphylococcus epidermidis*, Langmuir isotherm, liposomes.

1 INTRODUCTION

One unfortunate consequence of recent advances in therapeutic procedures is that the increased use of implanted biomedical devices such as catheters, prosthetic heart valves and artificial hip joints has led to a greater susceptibility to bacterial infection.[1] The organism most frequently isolated from such infections is *Staphylococcus epidermidis*,[2] which is normally a nonpathogenic organism associated with human skin.[2] Although harmless to a normal individual, this bacterium can cause disease when it persists in sites inaccessible to host defences or if these mechanisms are defective. The foreign body biomaterials of implanted devices interfere with the methods the body normally uses to deal with bacterial infection (e.g. leukocyte phagocytosis). Most of the *S. epidermidis* infections are of a nosocomial origin; that is, they originate and spread within the hospitals[3–5] and the skin around the insertion site for the device is considered the most common source of microorganisms that cause these infections. Once the skin barrier has been breached, the bacteria infect the devices by adhering to and colonizing their biomaterial surfaces[6] where they secrete an extracellular polysaccharide (slime) which forms a matrix.[7] The bacteria become embedded in this slime, forming a thick biofilm.[8] In biofilms, the bacteria have a lower metabolic activity and so are less susceptible to antimicrobial agents. The biofilm also acts as a penetration barrier.

Device-associated bacterial infections can lead to serious consequences, most frequently septicaemia. Replacement of the infected device is often the only option. As well as being potentially fatal, such infections can be costly; it has been estimated that replacement of an infected device increases the total cost of the operation by 400–600%.[1] Therefore, it is important to take

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preventative measures (e.g. cleansing of the insertion site) before implantation. Where infection has taken place, antibiotics can be used to treat the patient, particularly vancomycin.[9] However, vancomycin is potentially toxic and cannot be given orally. Intravenous administration is required over several weeks, with frequent monitoring of blood to ensure non-toxic levels of the drug.

Phospholipid vesicles (liposomes) have been shown to interact with bacteria[10–12] and can be used as vehicles for delivery of antibacterial agents. Targeting of liposomes to bacterial surfaces increases the efficiency of the encapsulated agent and reduces its general distribution, thus decreasing side effects. Cationic liposomes, incorporating positively charged stearylamine (SA), have been shown previously to target to biofilms of *S. epidermidis*,[13] exploiting the negative charge on the surface of the bacteria. Such vesicles could be used to deliver agents such as vancomycin to the bacteria, hopefully minimizing the unwanted side-effects of the free drug.

We describe here the targeting of cationic liposomes to *S. epidermidis* bacteria in the form of adsorbed biofilms. The interaction is studied in terms of liposomal lipid concentration and the effect of ionic strength of the media on targeting is examined, giving insights into the nature of the interaction.

2 MATERIALS AND METHODS

L-α-dipalmitoylphosphatidylcholine (DPPC, product No. P0763), cholesterol (Chol; product No. C8667) and stearylamine (SA; product No. S9273) were from Sigma Chemical Company, Poole, Dorset, UK. [^{3}H]DPPC (specific activity 55 Ci mmol^{-1}) was from Amersham International, Amersham, UK. Bacteriological agar No. 1 (code L11), brain heart infusion (BHI, code CM255), yeast extract powder (code L21) and phosphate-buffered saline (PBS) tablets (code BR14a) were from Oxoid Ltd, Basingstoke, Hants, UK. All other reagents were of analytical grade and aqueous solutions were made up with double-distilled water.

2.1 Preparation and characterisation of liposomes

Liposomes were prepared using the vesicle extrusion technique (VETs).[14] DPPC (27 mg), cholesterol (7 mg) and stearylamine (4 mg), together with [^{3}H]DPPC (5 μCi), were dissolved in *tert*-butyl alcohol (20 ml) in a 100-ml round-bottomed flask. The solvent was removed by rotary evaporation at 60°C to leave a thin, even lipid film to which was added PBS buffer (3 ml, pH 7·4) at 60°C. For the preparation of VETs, the suspension was vigorously mixed on a vortex mixer to form multilamellar liposomes (MLVs) followed by extrusion 10 times at 60°C though two stacked polycarbonate filters under a pressure of 1.4×10^6–2.8×10^6 N m^{-2}.

The lipid content was determined by scintillation counting of [^{3}H]DPPC. Size measurements were made by photon correlation spectroscopy (PCS) using a Malvern Autosizer model RR146. The scattering data were fitted to an equivalent normal weight distribution $W(d_i)$ to give the weight average diameter ($\bar{d}_w$).

2.2 Preparation of bacterial cultures

Staphylococcus epidermidis bacteria (NCTC 11047) were obtained from the University of Manchester collection. They were used to inoculate agar plates prepared from BHI (3·7 g) in double-distilled water (100 ml) to which was added bacteriological agar (1·5 g). The plates were inoculated by streaking and the inverted streaked plates were incubated at 37°C for 18 h. The resulting colonies were used to inoculate aliquots (10 ml) of nutrient broth prepared by mixing BHI (3·7 g) and yeast extract powder (0·3 g) in double-distilled water (100 ml). These were incubated in capped bottles at 37°C for 18 h after which the bacterial suspensions were centrifuged (2000 rev min^{-1}; 15 min), the supernatant discarded and the pellet resuspended in sterile PBS. The centrifugation and resuspension were repeated a further three times and the bacterial concentration adjusted to give an absorbance of 0·5 at 550 nm.

2.3 Adsorption of liposomes (VETs) to bacterial biofilms (Targeting assay)

Targeting assays were carried out in wells of microtitre plates (Dynatech M129B). Aliquots of bacterial suspension were incubated overnight at room temperature to form an adsorbed biofilm. After adsorption, the bacterial suspension was removed and the biofilm washed three times with sterile PBS. The adsorbed biofilms in the wells were then incubated with a liposome suspension (2 h, 37°C) after which the wells were washed three times with PBS and the biofilm dispersed by addition of sodium dodecylsulfate (SDS, 10 g litre^{-1}; 200 μl) followed by incubation (30 min) at room temperature and a brief sonication (2 min). Aliquots of the dispersed biofilm (180 μl) were taken for scintillation counting. Control wells containing only bacteria, only PBS or only liposomes were used to assess background levels of activity.

The results of the targeting assays are expressed in terms of the percentage apparent monolayer coverage (%amc) given by

$$\%\text{amc} = \frac{N_{\text{obs}}}{L_a} \times 100 \tag{1}$$

where N_{obs} is the observed number of moles of lipid adsorbed to the biofilm and L_a the number of moles of lipid which would be adsorbed if the biofilm was covered with a close-packed layer of liposomes. L_a was calculated from the equation

$$L_a = \frac{A_{bf}\bar{N}_w}{\pi(\bar{d}_w/2)^2} \qquad (2)$$

where $\bar{d}_w$ is the weight average diameter of the liposomes having a weight average number of moles of lipid per liposome of $\bar{N}_w$ and A_{bf} is the geometric area of the biofilm. $\bar{N}_w$ was calculated from $\bar{d}_w$ assuming an area per lipid molecule in the liposomal bilayer (taken as 0·5 nm^2) and a bilayer thickness (taken as 7·5 nm) as described previously.[15] The area of the biofilm was taken as $2{\cdot}202 \times 10^{-4}$ m^2 which was measured in a previous study for the surface of microtitre plate wells exposed to 200 μl of solution.[16]

Targeting assays performed at ionic strengths higher than that of the stock PBS solution were done by diluting the liposomes in high ionic strength solutions made by using more than the one PBS tablet per 100 ml distilled water used normally. Increasing the PBS concentration did not alter the pH.

2.4 Reversibility of adsorption of liposomes to biofilm

Liposomes (200 μl; 0·51 mM lipid concentration) in PBS buffer were incubated with bacterial biofilms in microtitre plate wells for two hours at 37°C. The plate was then washed three times with sterile PBS. After washing, the biofilm and adsorbed liposomes were incubated with solutions of increasing ionic strength for a further two hours at 37°C, then washed again with PBS and the amount of adsorption measured, as detailed in Section 2.3.

3 RESULTS

3.1 Liposome characterisation

The size distribution of a population of the cationic DPPC/cholesterol/SA liposomes is shown both before (Fig. 1) and after (Fig. 2) the extrusion process. Before extrusion the liposomes were multilamellar (had more than one lipid bilayer) and covered a wide range of sizes. After extrusion the liposomes were unilamellar VETs with a relatively homogenous population in terms of size, with weight average diameters of 127·6 nm reflecting the size of the pores of the polycarbonate filters used (100 nm). Scintillation counting before and after extrusion showed that 95·4 ($\pm$0·6)% of the lipid

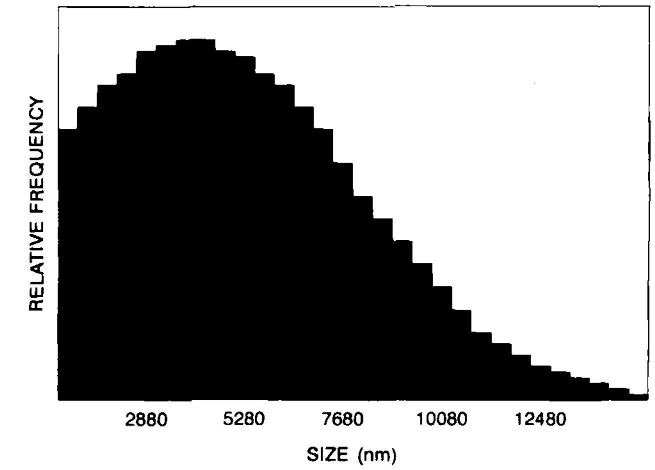

Fig. 1. Size distribution of a sample of multilamellar vesicles (MLVs), composition DPPC/Chol/SA (molar ratio 1:0·4:0·49).

from the multilamellar populations ended up in the unilamellar liposomes.

3.2 Dependence of adsorption of liposomes on lipid concentration

The adsorption of the DPPC/Chol/SA liposomes to *S. epidermidis* biofilms was studied as a function of liposome concentration (Fig. 3). The level of stearylamine used in the liposomes (21·3 mol%) had been identified previously as that which gave optimum targeting to *S. epidermidis*.[13] The %amc rises steeply between a liposomal lipid concentration of 0 and 1 mM and more slowly thereafter. Concentrations in the region of 0·3–0·4 mM are sufficiently high to give adsorption levels which were easily measurable. Neutral liposomes composed of DPPC/Chol with no SA did not adsorb to the bacteria significantly, giving %amc values of less than 5% at all the liposomal concentrations studied.

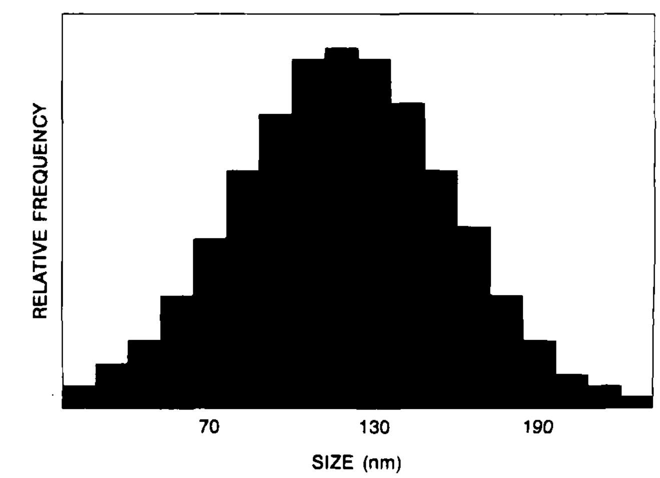

Fig. 2. Size distribution of a sample of vesicles produced by extrusion techniques (VETs), composition DPPC/Chol/SA.

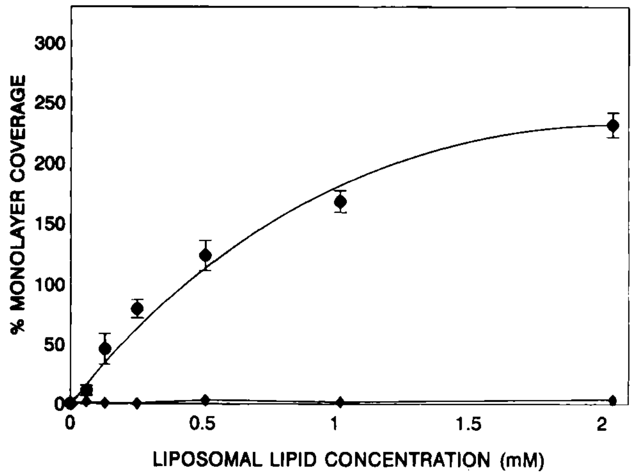

Fig. 3. Dependence of adsorption of liposomes to *S. epidermidis* biofilms on liposomal lipid concentration in PBS at 37°C. Liposome compositions were (●) DPPC/Chol/SA; (◆) DPPC/Chol.

3.3 Dependence of targeting on ionic strength of media

The concentration dependence of adsorption was studied at three higher ionic strengths, as well as in the standard PBS buffer. Over the range of liposomal concentrations studied, the %amc decreased when the ionic strength was increased (Fig. 4). The highest levels of adsorption were obtained with PBS at 180 mM, and the lowest levels at the highest ionic strength studied (666 mM).

3.4 Reversibility of adsorption of liposomes to the biofilm

Reversibility of adsorption was tested by comparing incubation of the liposomes (0·51 mM lipid concentration) in solutions of increasing ionic strength, with adsorption of liposomes from PBS solution (ionic strength 180 mM) followed by incubation with solutions of increasing ionic strength. Both sets of experiments gave comparable results, demonstrating the reversibility of adsorption (Fig. 5).

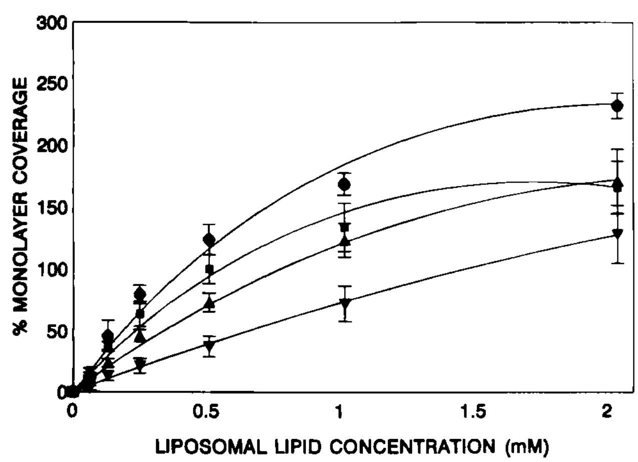

Fig. 4. Dependence of adsorption of DPPC/Chol/SA liposomes to *S. epidermidis* biofilms on ionic strength of the media at 37°C. The ionic strengths were (●) 180 mM; (■) 342 mM; (▲), 504 mM; (▼) 666 mM.

4 DISCUSSION

Adsorption of SA-containing liposomes to biofilms of *S. epidermidis* was found to be dependent on liposomal lipid concentration (Fig. 3). The %amc increased with liposome concentration, giving values of well over 100. The biofilms were formed by adsorption of bacteria from suspensions which had an absorbance of 0·5 at 550 nm, leading to a close-packed multilayer of cells that can be visualized by electron microscopy.[17] It should be noted that the values of %amc over 100 do not necessarily imply that the liposomes are forming a multilayer on the biofilm. The calculation of %amc is made with reference to the geometric surface area of the biofilm (microtitre plate well) and, depending on the surface roughness of the biofilm, will to some degree overestimate the coverage. For example, if the bacteria were represented as smooth hemispheres on the surface of microtitre plate then their surface area would be doubled and hence the %amc halved. Hemispheres with a rough surface would have a larger surface area and the %amc would be reduced further.

The reversibility of adsorption (Fig. 5) is consistent with an equilibrium between adsorbed and bulk-state liposomes. This being so, the extent of adsorption as a function of liposomal concentration can be described in terms of a Langmuir adsorption isotherm.[18] If $\varnothing$ is the fraction of the surface area that is covered by adsorbed

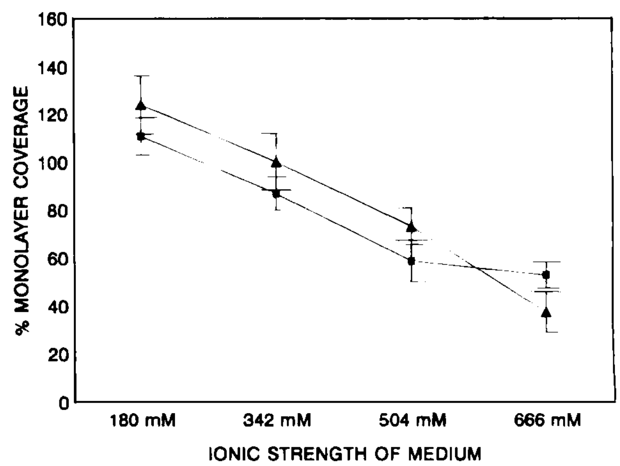

Fig. 5. Reversibility of the interaction between DPPC/Chol/SA liposomes (0·51 mM) to *S. epidermidis* biofilms. (▲) Adsorption of liposomes as a function of ionic strength of the medium. (■) Adsorption of liposomes after initial adsorption from PBS (180 mM) followed by incubation as a function of ionic strength of the medium.

liposomes in equilibrium with liposomes, concentration C, in the bulk phase then the Langmuir adsorption isotherm may be written.

$$\varnothing = \frac{C}{K_d + C} \tag{3}$$

where K_d is the dissociation constant and $\varnothing$ is given by (%amc)/(%amc)$_{max}$, where (%amc)$_{max}$ is the maximum apparent monolayer coverage. Substituting for $\varnothing$ in eqn (3) and reciprocating gives the linear form of the isotherm;

$$\frac{1}{\%\text{amc}} = \frac{1}{C}\frac{K_d}{(\%\text{amc})_{max}} + \frac{1}{(\%\text{amc})_{max}} \tag{4}$$

from which K_d and (%amc)$_{max}$ can be obtained from the slope and intercept of the plot of 1/%amc against $1/C$.

The double-reciprocal plots of the results are shown in Fig. 6 and the values of (%amc)$_{max}$ and K_d are in Table 1, together with the standard Gibbs energies of dissociation ($\Delta G_d^\circ = -RT \ln K_d$). These results show that, as the ionic strength is increased, the dissociation constant increases, but also that (%amc)$_{max}$ is independent of ionic strength within the experimental error. The average value of (%amc)$_{max}$ is 300 (±15)%. If the true maximum surface coverage is 100%, this figure suggests that the surface area of the biofilm is approximately three times the geometric surface area.

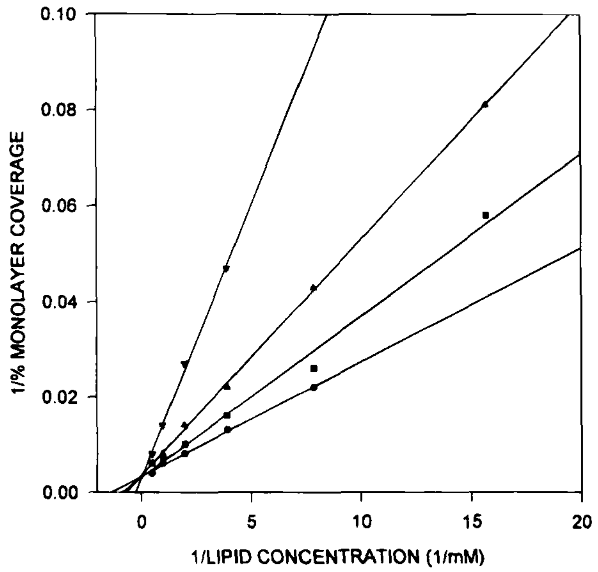

Fig. 6. Double-reciprocal plot of the data in Fig. 4, [1/%amc] *vs.* 1/[liposomal lipid].

The results indicate that the attractive interaction between the cationic vesicles and negatively-charged sites on the bacterial surface (e.g. teichoic acids) is mediated by electrical double-layer effects, in that compression of the diffuse double layer and increased ionic screening at higher ionic strength weakens the interaction between bacterium and liposome. The change in ionic strength does not affect the (%amc)$_{max}$ because on extrapolation of the liposomal concentration to infinity, the ionic strength effect is abolished.

The interaction between the vesicles and *S. epidermidis* is reversible. When liposomes (0·51 mM solution) were incubated in PBS buffer, followed by washing and addition of solutions of higher ionic strength, the %amc fell from 124 to levels similar to those found when the liposomes were initially applied in these high ionic strength solutions (Fig. 5). The results demonstrate that a new position of equilibrium between liposomes in the bulk state in solution and adsorbed liposomes is established at each ionic strength.

The screening of ionic interactions is related to the thickness of the ionic atmosphere associated with the bacterium and liposome surfaces. The reciprocal of the Debye–Hückle parameter, κ, is generally taken as a measure of the thickness of the ionic atmosphere and is related to ionic strength (I) for a symmetrical electrolyte by the equation[19]

$$\frac{1}{\kappa} = \left(\frac{\varepsilon_0 \varepsilon_r kT}{2N10^3 e^2}\right)^{1/2} \frac{1}{\sqrt{I}} \tag{5}$$

where N, e, ε_0, ε_r, k and T are Avogadro's constant, electronic charge, permittivity of vacuum, relative permittivity of the medium, Boltzmann constant and temperature, respectively. It follows that, for an electrostatic interaction, adsorption would be expected to decrease as the ionic strength increases and the ionic atmosphere is compressed. Thus the adsorption constant K_a ($=1/K_d$) might be expected to be related to the

TABLE 1

Values of K_d, (%amc)$_{max}$ and Standard Gibbs Energies of Dissociation (ΔG_d°) for the Interaction between the Cationic Liposomes and the Bacterial Biofilm, as derived from Fig. 6

Ionic strength of medium (mM)	K_d *(mM liposomal lipid)*	*(%amc)$_{max}$*	*Gibbs energy of dissociation (kJ mol^{-1})*
180	0·729 (±0·061)	303·3 (±24·2)	+18·6
342	1·015 (±0·127)	294·1 (±36·6)	+17·8
504	1·403 (±0·162)	283·6 (±32·5)	+16·9
666	3·628 (±1·340)	319·4 (±117·1)	+14·5

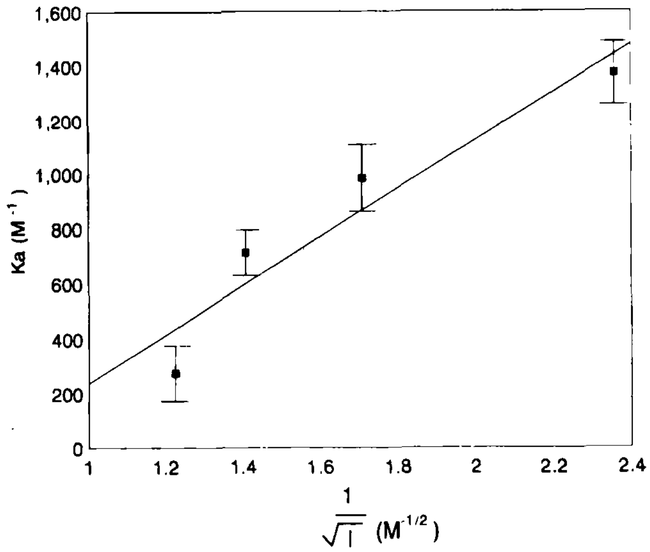

Fig. 7. Plot of the association constant (K_a) against the reciprocal of the square root of the ionic strength of the media.

thickness of the ionic atmosphere ($1/\kappa$) or $1/\sqrt{I}$. Figure 7 shows the plot of K_a *vs.* $1/\sqrt{I}$ in which K_a decreases linearly with increasing $\sqrt{I}$. The intercept on the x-axis when $K_a = 0$ is 0·73, corresponding to an ionic strength of approximately 1·9 M, at which no adsorption would occur.

These results demonstrate the strong affinity of stearylamine-containing liposomes for *S. epidermidis* bacteria. The interaction seems to be of an electrostatic nature and involves negative charges associated with the bacterial biofilm—either on the bacteria or the surrounding biofilm. Such liposomes should be ideal vehicles for delivery of antibacterial agents. Work needs to be done on the encapsulation of vancomycin, which is water-soluble. Due to the presence of a bacterial cell wall, fusion between vesicle and bacteria is unlikely; it is more probable that adsorbed liposomes will allow for slow and sustained release of the drug at the site of infection. The high-ionic-strength-induced removal of liposomes from the bacterial surface could be used to examine the passage of such encapsulated agents between the liposome and the bacteria. If such passage does occur, removed vesicles should have a lower concentration of encapsulated agent than when they were initially applied, and there should be a larger amount of the drug associated with the bacteria than could be accounted for by the quantity encapsulated with the vesicles still adsorbed.

One possible problem with these cationic liposomes is the toxicity associated with stearylamine.[20] To overcome this problem, an alternative cationic molecule, dimethyldioctadecylammonium bromide (DDAB), has been used in the liposome formulation, and early results indicate that such liposomes will be just as effective as the stearylamine liposomes in adsorbing to biofilms of *S. epidermidis*.

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